REMARKS

In view of the above amendments and the following remarks, the Examiner is respectfully requested to withdraw the rejections, and allow claims 1, 2, 6-8, 10 and 12-13, the currently considered claims. No new matter is added.

An additional copy of reference AB, Brossay *et al.* (1998) Immunological Reviews 163:139-150, as requested by the Examiner, is attached herewith.

A correction to the Brief Description of the Drawings is provided herewith.

The Office Action states that the restriction requirement has been made final. Applicants reserve the right set forth in 37 C.F.R. 1.114 and M.P.E.P. 818.03(c) to petition the Commissioner for review of the requirement, which petition may be deferred until after final action on or allowance of claims to the invention elected.

The Office Action states that Applicants should amend the first line of the specification to update the status and relationship of the priority documents. Applicants respectfully draw the Examiner's attention to the Application Data Sheet (ADS), which was filed April 27, 2002, and which provides the priority claim to U.S. Provisional Application 60/200,285. As set forth in 37 C.F.R. 1.76 (b)(5), *Domestic priority information*: "This information includes the application number, the filing date, the status (including patent number if available), and relationship of each application for which a benefit is claimed under 35 U.S.C. **119(e)**, **120**, **121**, or **365(c)**. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. **119(e)** or **120**, and **§ 1.78(a)(2)** or **§ 1.78(a)(4)**, and need not otherwise be made part of the specification." Withdrawal of the objection is requested.

Claims 1, 2, 4-7, 9 12 and 13 have been rejected under 35 U.S.C. 112, first paragraph. The Office Action states that the instant claims encompass treatment of patients with a CD1 blocking agent of undisclosed structure and/or specificity and a second agent of undisclosed structure.

Applicants respectfully submit that the presently claimed invention meets the requirements of 35 U.S.C. 112, first paragraph. Without conceding to the correctness of the rejection, in order to further prosecution, independent Claim 1 has been amended to recite the

use of an antibody that binds to CD1. Such antibodies are clearly defined in the specification, and examples of the use of such antibodies are provided in the present application.

With respect to the second therapeutic agent, Applicants have specified the use of an immunosuppressant, anti-inflammatory, or anti-coagulant agent. Applicants respectfully submit that it is commonplace to claim combination therapies with known, therapeutic agents, and it is not required that Applicants name specific agents that find use in such methods. As described in the specification, a number of specific agents are known and used in the art for the treatment of SLE. One of skill in the art can readily combine treatments, and such combinations require no more than routine experimentation.

In view of the above amendments and remarks, withdrawal of the rejection is requested.

Claims 1, 2, 4-8, 10 and 12 have been rejected under 35 U.S.C. 103(a) as unpatentable over Amano *et al.* in view of Kotzin *et al.*, Zeng *et al.*, Blumberg *et al.* and Hughes. Applicants respectfully submit that the presently claimed invention is not made obvious by the cited combination of references. Applicants have provided actual *in vivo* evidence that blocking CD1 by administration of antibodies significantly reduced the peak levels of serum IgG and IgG anti-dsDNA autoantibodies, and delayed disease progression. Further, these results were obtained with a spontaneous disease model, in contrast to the prior art model, which required transfer of cells. It may be noted that human lupus occurs spontaneously.

Although the art suggested a possible connection between lupus and CD1, there was substantial uncertainty that CD1 had a causative role, or was merely associated with the disease in these systems. Without the findings provided in the present application, one of skill in the art could not have a reasonable certainty of success practicing the claimed methods.

Amano *et al.* identify two subpopulations of splenic B cells that express high levels of a ß2m-dependent form of CD1: marginal zone B cells and a newly defined population of follicular (IgDhigh CD23+) B cells. Flow cytometric analysis demonstrated that 70 to 75% of CD1high B cells are marginal zone B cells. These cells expressed high levels of IgM, low levels of IgD, and lacked expression of CD23[1].

Amano *et al.* go on to suggest that T cell recognition of CD1 on the surface of B cells might play a role in the pathogenesis of systemic lupus. The basis for this assertion is the finding that $CD4^+$ and $CD8^+$ T cells expressing anti-CD1 TCR transgenes obtained from a

[1] page 1715, first full paragraph.

Vß9/V4.4 T cell clone will induc lupus when transferred into syngeneic BALB/c nude hosts. Amano *et al.* then cite the Zeng *et al.* (1998) paper to support this position[2].

A major drawback of the work published by Amano *et al.*, and Zeng *et al.*, is the use of a transgenic animal model, which requires transferring disease causing T cells into an immune compromised recipient. In this model, transgenic animals were created which had transgenic T cell receptors that recognized CD1. Specific sorted populations of these T cells were transferred to recipient animals, which were then assessed for disease. In these animals, virtually all the T cells present in the host expressed the T cell receptor transgene.

However, although the transgenic T cells induced overt lupus in adoptive nude hosts, the **transgenic donor mice did not develop lupus nor did adoptive euthymic hosts**[3]. It was speculated that T cells or other thymus-dependent regulatory cells might inhibit the development of disease.

Further, Zeng *et al.* state: "BM cells containing SP transgenic $CD4^+$ and $CD8^+$ T cells induced lupus in most nude recipients, but **BM cells from DN transgenic mice containing transgenic $CD4^+$, $CD8^+$, and CD4CD8 T cells failed to induce lupus in any recipients**. Mixing experiments showed that **the latter BM cells ameliorated lupus disease abnormalities** induced by SP transgenic BM cells. Sorted transgenic CD4CD8 T cells derived from the DN BM were very effective in preventing disease induced by SP BM cells, and none of the hosts given a combination of these cells developed proteinuria during the 100-d observation period."[4]

Therefore, the data presented by Zeng *et al.* (1998) demonstrate that animals transgenic for a T cell receptor that recognizes CD1 do not develop disease; and that certain populations of the T cells can be transferred to cause disease, while other populations of T cells suppress disease. From these findings, one of skill in the art could not conclude with any degree of certainty that CD1 would have a causative effect in lupus.

The *in vivo* data provided in the instant application was obtained in an animal model for lupus where the disease is spontaneously induced, and therefore does not suffer from the issues raised above for transfer of transgenic T cell subsets.

Applicants respectfully submit that the secondary references do not remedy the deficiencies of the primary references. Blumberg *et al.* teaches the expression of CD1 on B

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[2] Reference 33 of Amano *et al.*

[3] Zeng et al., page 533, second column.

[4] Zeng et al., page 533, last paragraph.

cells, monocytes and Langerhans cells, but fails to demonstrate the effectiveness of blocking CD1 to treat lupus-like disease.

Hughes provides background for the use of antibodies as therapeutics, but fails to teach the usefulness of antibodies specific for CD1 in the treatment of lupus-like disease.

Kotzin reviews the pathology of lupus, in particular the clonal expansion of anti-DNA antibody-producing B cells. However Kotzin fails to teach an association of CD1 with the disease, and does not show the effectiveness of blocking CD1 to treat lupus-like disease.

Claim 13 has been rejected under 35 U.S.C. 103(a) as unpatentable over Amano *et al.* in view of Kotzin *et al.*, Zeng *et al.*, Blumberg *et al.* and Hughes, further in view of the Merck Manual. Applicants respectfully submit that the invention of Claim 13 is not made obvious by the cited combination of references. As discussed above, the prior art does not provide a reasonable expectation that administration of CD1 would be effective in treating lupus-like disease. The inclusion of a second therapeutic regimen is not relied upon for patentability, but is merely put forth as a variation on Applicants methods.

In view of the above amendments and remarks, Applicants respectfully submit that the present claims meet the requirements of 35 U.S.C. 103. Withdrawal of the rejections is requested.

Conclusion

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number STAN-190.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

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Antigen-presenting function of mouse CD1: one molecule with two different kinds of antigenic ligands

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Summary: Mouse CD1 (mCD1) is an antigen-presenting molecule that is constitutively expressed by most bone marrow-derived cells. Peptides with a hydrophobic binding motif can bind to mCD1, and the peptide-CD1 complex is recognized by $CD8^+$ cytolytic T cells. In contrast, $NK1.1^+$ T cells, which are $CD8^-$, are autoreactive for mCD1 molecules. This autoreactivity, along with the ability of NK T cells to rapidly produce large amounts of cytokine, has led to the suggestion that these cells may be immunoregulatory. We have shown that the mCD1-autoreactive T cells can distinguish between different cell types that express similar levels of mCD1, suggesting that mCD1-bound autologous ligands may be critical for T-cell stimulation. Consistent with this, some of these mCD1-restricted T cells can recognize the glycolipid α-galactosylceramide presented by mCD1, while others do not respond. The mCD1 crystal structure reveals a deep and narrow hydrophobic antigen-binding site which can more easily bind lipid antigens than the long hydrophobic peptides that we have defined as mCD1 antigens. The ability of mCD1 to bind and present two different types of ligands raises the question as to how mCD1 can accommodate both types of antigens.

Introduction

This review focuses on recent data implicating mouse CD1 (mCD1) as an antigen-presenting molecule. We and others have recently published reviews on related topics, and we recommend these articles to readers interested in NK T cells (1–4) and T-cell recognition of mouse and human CD1 molecules (5–12).

The mouse cluster of differentiation 1 (CD1) gene family consists of two non-polymorphic genes, *mCD1D1* and *mCD1D2*, mapped to a cluster on chromosome 3. They are highly related to one another and, compared to the genes encoding the four human CD1 proteins, they are most similar to the human *CD1D* gene in sequence. CD1 glycoproteins have the typical structure of antigen-presenting class I molecules. They are heterodimers consisting of an approximately 50 kDa glycosylated heavy chain associated non-covalently with the β2-microglobulin (β2m) light chain. The heavy chains are type I transmembrane glycoproteins with an extracellular portion formed by α1, α2 and α3 domains of 90 amino acids each, a transmembrane domain and

a very short cytoplasmic tail (reviewed in (6)). Despite this overall similarity to class I molecules, comparisons of the primary sequence demonstrate that CD1 molecules are about as closely related to class II molecules as they are to class I molecules (13), and therefore they probably diverged from these other antigen-presenting molecules early in vertebrate evolution around the time of the class I-class II divergence.

One of the most striking differences which clearly distinguishes CD1 molecules from MHC-encoded classical class I and class II molecules is the absence of a significant degree of allelic polymorphism of CD1 proteins (14). The lack of CD1 polymorphism raised the hypothesis that these proteins may not be effective antigen-presenting molecules (15). In fact, growing evidence suggests that CD1 molecules, as well as some other non-polymorphic class I molecules, have specialized functions in the presentation of antigens with limited structural variability (16–22). Unlike several of the MHC-encoded non-polymorphic class I molecules, CD1 can be found in a variety of mammals (23–27). As a result of their early divergence from class I and class II molecules, their presence in a number of different species, and their unique antigen-presenting properties (see below), CD1 molecules are believed to constitute a separate, third category of antigen-presenting molecules.

In this review, we will focus nearly entirely upon CD1 molecules in the mouse, including a comparison of the behavior of mCD1 with MHC-encoded class I and class II molecules, a description of the T cells specific for mCD1, a discussion of the ability of mCD1 to present two chemically different kinds of antigens, and some speculations on the evolution of CD1 molecules and the significance of CD1-mediated immune responses.

Tissue distribution of mCD1

mCD1 was originally thought to be expressed mainly by intestinal epithelial cells, and therefore it was to be a candidate antigen-presenting molecule recognized by intraepithelial lymphocytes (IEL) (28). Although the expression of mCD1 on epithelial cells from the intestine remains a controversial issue, our data demonstrate that mCD1 is in fact found mainly on the surface of hemopoietic lineage cells (29). Furthermore, in contrast to the known pattern of expression of the human CD1a, CD1b and CD1c molecules, mCD1 is constitutively expressed by bone marrow-derived cells in the mouse, including B cells, T cells, macrophages and dendritic cells. We have not found any mCD1-negative T cells or B cells in central or peripheral lymphoid tissues such as thymus, bone marrow, lymph node, spleen, blood stream, gut-associated lymphoid tissues and IEL. The level of expression of mCD1, however, can vary within a lineage. For example, while dendritic cells derived from bone marrow express a moderate amount of mCD1 (29), dendritic cells freshly isolated from the spleen express high levels of mCD1 on their surface (Fig. 1). Similarly, heterogeneity of mCD1 expression on mouse B cells also has been reported, with marginal zone B cells of the spleen and a subset of B cells in the peritoneal cavity having the very highest levels of mCD1 (30). Transformed cell lines show a greater heterogeneity in the level of mCD1 expression, although such heterogeneity may not have an in vitro correlate. For example, within the B-cell lineage, A20 cells do not express detectable levels of mCD1, while BCL1 cells express the highest levels we have detected on any cell (Fig. 1). In addition to expression by hemopoietic lineage cells, in agreement with an earlier report (28), it was found that mCD1 also is expressed by hepatocytes and a transformed hepatocyte cell line. The relatively high level of expression of mCD1 in bone marrow-derived cells, and mainly on professional antigen-presenting cells such as dendritic cells, macrophages and B cells, is consistent with a possibly important antigen-presenting function for mCD1 in host defense and/or immune regulation. Furthermore, the abundant expression of mCD1 in thymus, bone marrow, liver and spleen correlates with the relatively high frequency of mCD1-autoreactive NK T cells in these sites (1, 31).

Properties of mCD1

TAP independence and β2m dependence of mCD1 expression

Classical class I molecules can only be expressed in a stable conformation at the surface of the cell if the intracellular heavy chain has bound β2m and peptide has been loaded in the antigen-binding groove (32). The interaction of the class I heavy chain with these two components occurs in the endoplasmic reticulum (ER) of the cell prior to transport to the cell surface. The major supply of class I-binding peptides is transported from the cytoplasm to the ER by the transporter associated with antigen processing (TAP) molecule, an ATP-dependent peptide transporter located in the ER membrane. The TAP molecule is a heterodimer encoded by two MHC-encoded genes, *TAP-1* and *TAP-2*. Cells which lack one or both of the TAP gene products are not able to stably express class I molecules at the cell surface (33, 34). In contrast, TAP-deficient cells such as mCD1-transfected *Drosophila melanogaster* cells and RMA-S cells do express mCD1 at the cell surface (35, 36). In addition, both the tissue distribution and the level of expression of mCD1 are comparable in TAP-1-deficient mice and in wild-type mice (29, 37)

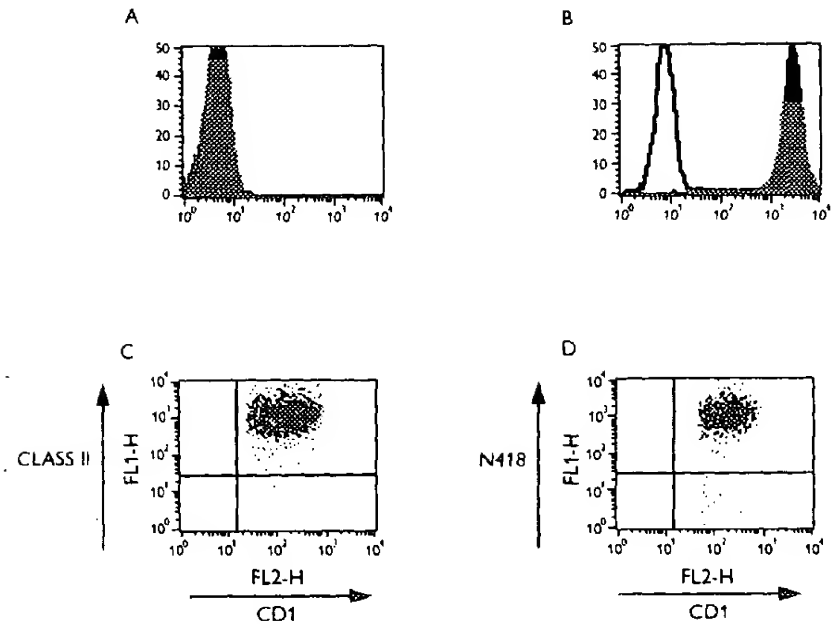

Fig. 1. Heterogeneity of mCD1 expression by hemopoietic lineage cells. Flow cytometry profiles of untransfected A20 (A) and BCL1 B cells (B) are shown. Stainings were carried out with 1B1-PE mAb. Before each staining, cells were incubated with anti-Fcγ receptor mAb 2.4G2. Each panel is an overlay of the isotype control (open histogram) and the anti-CD1 mAb (solid histogram). (C) Freshly isolated dendritic cells (97) express high levels of mCD1. Splenic tissue was digested with collagenase type III, and, following a Percoll gradient, the low-density fraction was allowed to adhere on plastic for 90 min at 37°C. Adherent cells were cultured overnight, and the non-adherent cells in the overnight culture were recovered and stained. These cells have the characteristic pattern of expression of high levels of class II, they are N418-positive, and they are FcγR-low. Two color flow cytometry profiles for dendritic cell expression of class II and mCD1 (C) and N418 and mCD1 (D) are shown.

(L. Brossay, unpublished data). This set of data is consistent with the fact that all the mCD1-reactive T cells described so far, including the NK T cells and the peptide-specific CD8 T cells, have been found in TAP-deficient mice (18, 36) (N. Burdin & S. Tangri, unpublished data). In contrast, these mCD1-reactive T cells are found neither in β2m-deficient mice nor in CD1-deficient mice (38–44).

Although the interaction of β2m and the heavy chain stabilizes CD1 molecules at the cell surface (45, 46), there is a report of β2m-independent expression of mCD1 in transfected, β2m-deficient, FO-1 human melanoma cells (47) - however, this result is controversial (36). We could not detect any significant level of surface expression in mCD1-transfected β2m-deficient cells such as RIE (35) or Daudi cells (*Fig.* 2). However, the expression of mCD1 is detectable when the same Daudi cells are transfected with a full-length single chain version of the mCD1 which has the β2m covalently linked to the amino terminus of the mCD1 heavy chain via a glycine- and serine-rich peptide spacer (Fig. 2). Furthermore, the β2m domain of the single chain molecule does not rescue surface expression of endogenous class I molecules (L. Brossay, unpublished data). Finally, we could not detect significant levels of surface expression of mCD1 on any cell type tested from β2m $^{-/-}$ mice (Fig. 2). Several mCD1-reactive T-cell hybridomas, however, have been shown to be activated by cells that do not express β2m (48). This reactivity was poorly blocked by our mCD1 mAbs, suggesting that, as for classical class I molecules, any β2m-independent form of mCD1 on the cell surface is in a different conformation than the molecules found on wild-type, β2m$^+$ cells. By contrast to the results from β2m-deficient mice, studies with MHC class II-deficient mice have demonstrated that the CD1-restricted T cells, including the NK T cells, were present in normal numbers in these mice (48, 49).

mCD1 binds to CD8 molecules

We used a T-cell activation-based assay to show that mCD1 molecules can bind to mouse CD8 molecules (35), despite only a moderate degree of conservation of the major CD8-binding site found in the α3 domain of classical class I molecules. Given the results from the binding/activation assay, it was therefore not surprising to find CD8-positive, mCD1-restricted T-cell lines (see below). NK T cells, however, which are presumed to be mostly mCD1-autoreactive, are only rarely if ever CD8$^+$. These NK T cells also are absent in transgenic mice with forced expression of CD8 in all T-cell subpopulations, suggesting that expression of NK T-cell antigen receptors (TCRs) along with CD8 gives an overall avidity that leads to negative selection (39, 50).

In summary, although mCD1 is quite divergent in primary sequence from classical class I molecules, it shares at least two properties with class I molecules: a requirement for β2m for significant levels of surface expression in the native conformation and the ability to interact with CD8 molecules. In contrast, mCD1 is distinct from classical class I molecules in its lack of a requirement for TAP-derived peptides. These properties proba-

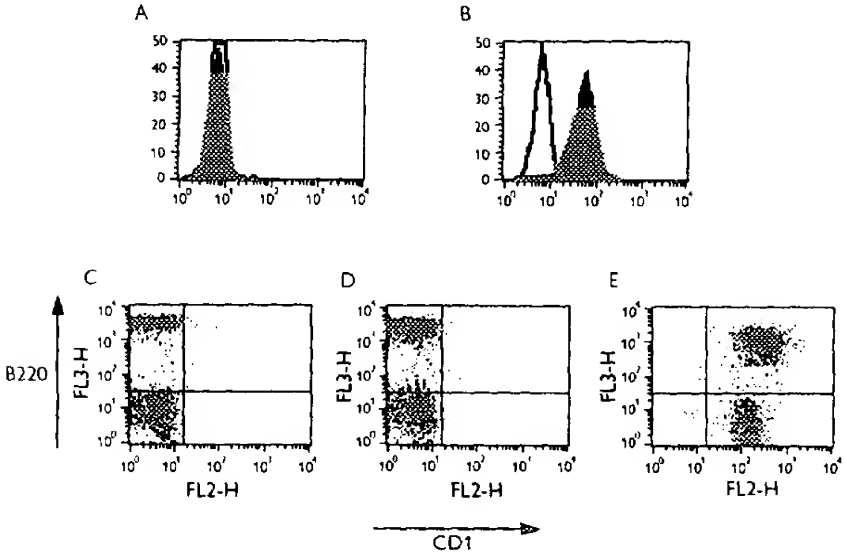

Fig. 2. Surface expression of mCD1 is mainly β2m-dependent. Flow cytometry profiles of (A) mCD1.1-transfected Daudi cells and (B) full-length, single chain β2m mCD1-transfected Daudi cells are shown. The technique to obtain chimeric single chain mCD1 molecules has been described for class I molecules by Lee et al. (98). Stainings were carried out with 1B1-PE mAb as described above. Each panel is an overlay of the isotype control (open histogram) and the anti-CD1 mAb (solid histogram). Splenocytes pooled from two mCD1 −/− mice (C), two β2m −/− (D) and two C57BL/6 mice (E) were incubated with 2.4G2 mAb and then stained with biotinylated anti-B220 and 1B1-PE mAb or isotype control, followed by streptavidin-tricolor.

bly are conserved among CD1 molecules, as human CD1a, b and c molecules also have been found to be TAP-independent (8, 51, 52) while they require β2m for surface expression (45).

mCD1 is directed to endosomes by an intracytoplasmic tyrosine-containing motif

The endosomal localization of CD1 is another feature which distinguishes these antigen-presenting molecules from class I proteins. Human CD1b molecules are found in a variety of endosomal structures, predominantly various types of late endosomes (53, 54). To define more precisely the intracellular distribution of mCD1, we used confocal microscopy to co-localize mCD1 with markers for endosomal compartments. We have recently demonstrated that mCD1 is present in endosomes, with extensive co-localization with late endosomal markers such as the mouse H-2M molecule (DM homolog). These data suggest that mCD1 is found in compartments specialized for class II peptide loading such as the lysosome-like MHC class II compartment (MIIC) and the class II-containing vesicles (CIIV) present in B cells and dendritic cells (55). This finding is confirmed here in a confocal scanning micrograph showing the extensive intracellular co-localization of mCD1 with class II molecules in A20 B-lymphoma transfectants (Fig. 3). Because the majority of the intracellular class II molecules in this cell line are found in the CIIV (56, 57), we speculate that this compartment also may serve as a specialized site for the loading of antigenic ligands onto mCD1 molecules. In an mCD1-transfected macrophage cell line (J774), we also show that mCD1 co-localizes with the late endosomal-lysosomal marker Lamp2 in internal vesicles (Fig. 3). Taken together, these sets of data demonstrate that mCD1 is found both in the CIIV and in lysosomes, as described for human CD1b.

We also determined if the localization of mCD1 in endosomal compartments is dependent upon its intracytoplasmic sequence. The intracytoplasmic domain of mCD1 has a tyrosine-containing motif, YQDI, which has been shown to be important for the trafficking of a variety of surface proteins into endosomes (58–63). In order to disrupt the endosomal localization of mCD1, we constructed several mutants in the mCD1 cytoplasmic tail. One has the COOH-terminal five amino acid sequence from the intracytoplasmic tail of mCD1 deleted (mCD1TD), and four others have single alanine substitutions of each of the amino acids from this putative endosomal localization motif. Neither the five amino acid deletion nor the single substitutions with alanine affected the ability of mCD1 to be expressed on the surface of transfected cells (55) (L. Brossay, unpublished data). Using confocal microscopy, we were able to show that permeabilized A20 cells expressing either the mCD1TD or the mCD1 Y→A mutants revealed homogenous staining around the rim of the cell that is characteristic of cell-surface staining (55). In the same study, we have also shown that the tail-deleted form of mCD1 does not co-localize with H-2M molecules, indicating a redistribution of mCD1 from endosomes to the cell surface. These data are consistent with those found for the human CD1b protein, which does not traffic to endosomes when the cytoplasmic tyrosine-containing sequence motif is deleted (53), and, furthermore, they also demonstrate that the tyrosine is critical for this process.

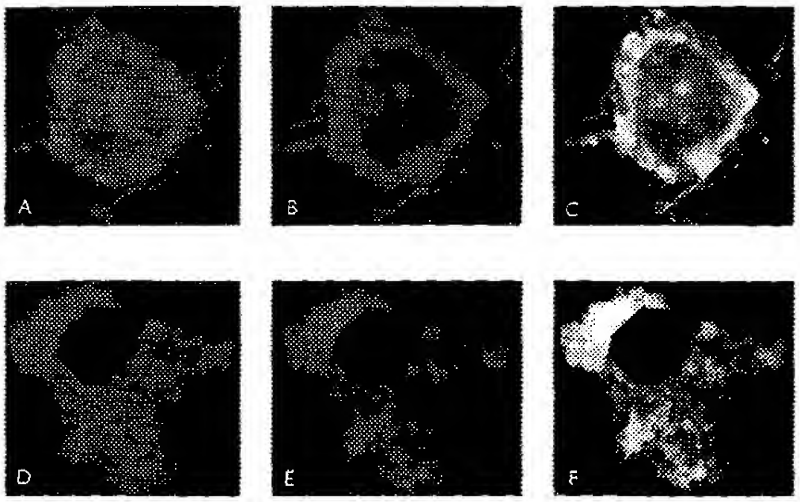

Fig. 3. mCD1 is found in lysosomes and late endosomes. After overnight adherence, mCD1 molecules were immunofluorescently localized in formaldehyde-fixed cells that were permeabilized with 0.2% saponin in PBS for 25 min. mCD1-transfected A20 cells (A, B and C) and mCD1-transfected J774 (D, E and F) were labeled with biotinylated 1B1 mAb, followed by either streptavidin coupled to FITC or streptavidin coupled to Texas-red. (A, B and C) mCD1 molecules (A, green) and class II molecules (B, red) were partially co-localized (C). (D, E and F) mCD1 molecules (D, red) and Lamp2 molecules (E, green) were co-localized (F).

mCD1-restricted T cells

mCD1 can present peptides

The ability of mCD1 to bind peptides with a hydrophobic sequence motif, and to present these peptides to T cells, has been clearly demonstrated by us and by others (18, 22). Using a codon-based random peptide library display, we were able to show that mCD1 could bind synthetic peptides with a hydrophobic motif that includes an aromatic residue at position 1 and 7, and an aliphatic residue at position 4. The potential immunologic relevance of these findings was established by raising peptide-specific mCD1-restricted T cells. The peptide recognized, p99, contains the motif, which was shown to be required both for mCD1 binding and for T-cell stimulation (*Table 1*). The peptide-reactive and mCD1-restricted T cells are TCR $\alpha\beta^+$ and CD8$\alpha\beta^+$, they are cytolytic and secrete IFN-γ, and they can be found in spleen and lymph nodes (18). Surprisingly, this sequence motif also is found in the hydrophobic N-terminal signal sequences of both mCD1 and hCD1d (1) (Table 1), where, hypothetically, it could be involved in the intracellular loading and subsequent trafficking of these CD1 molecules. Although tryptophan is a relatively rare amino acid, the mCD1-binding sequence motif also is found in a few other proteins from diverse organisms including eukaryotes, bacteria and especially viruses. A partial list of these proteins is presented in Table 1. Interestingly, the envelope glycoprotein (gp160) of the human immunodeficiency virus type 1 (HIV-1) contains this hydrophobic mCD1-binding motif (*Table 1*). Although peptide-specific, hCD1d-restricted, human T-cell lines have not been described, the development of peptide- and CD1-based vaccines is an attractive possible strategy on account of the lack of polymorphism of CD1 and its expression by hemopoietic cells. The potential of this strategy has been recently illustrated by the work of Lee et al. (22), who demonstrated that an mCD1-mediated CTL response could be elicited after immunization with a combination of naked plasmids DNA-encoding chicken ovalbumin, mCD1 and a co-stimulatory molecule. Chicken ovalbumin contains the mCD1-binding motif (*Table 1*) and the response to ovalbumin could be blocked by incubation with p99 (22), confirming the validity of our previous data (18).

Our recent data show that replacement of the endosomal localization signal of mCD1 causes this molecule to reach the cell surface without passing through endosomes, and that this mutation abolishes the ability to present ovalbumin peptide when antigen is given as whole ovalbumin protein externally to the antigen-presenting cells (S. Tangri, unpublished data). By contrast to the results with whole protein, the presentation of added ovalbumin 18-mer peptide is not affected by the mCD1 mutant. These results, and data obtained from antigen-presenting cells that have been fixed or treated with inhibitors, demonstrate that ovalbumin requires processing in order to generate the peptide epitope required by ovalbumin-specific T cells. Based upon these studies, we conclude that mCD1 traffics to endosomal compartments where it binds peptides derived from processed proteins.

Despite this clear evidence for both a physical interaction between mCD1 and peptide, and the presentation of different peptides by mCD1, no one has yet succeeded in identifying

peptides bound to immunopurified mCD1 molecules. Furthermore, it is not known how the relatively long (> 15 amino acid) peptides that are presented by mCD1 bind to it. mCD1 has a hydrophobic groove that is both deeper and narrower than the antigen-binding grooves of class I and class II molecules. The groove is not open-ended, as is the class II antigen-binding groove, raising the issue as to how the long peptides might fit into the molecule (63a). Finally, electron dense material found in the groove in the insect-derived material is not likely to be a peptide (I. Wilson, personal communication). Collectively, these data suggest that peptides may bind to mCD1 outside the groove, or in an unconventional way compared to the stable peptide/MHC complexes that class I and class II molecules form.

Table 1. Examples of some proteins with the mCD1-binding motif

Protein	Organism	Sequence - motif in bold
Glycoprotein K	Human herpes virus 2	RPL**FLTITTW**CPV
Envelope protein	Feline immunodeficiency virus	GSW**FRAISSW**KQ
Envelope protein gp160	HIV-1	KYL**WNLLQYW**SQE
Pilin gene-inverting protein	*Moraxella lacunata*	GAY**WEKLAHW**AIS
Ovalbumin	Chicken	IIN**FEKLTEW**TSS
mCD1.1	*Mus musculus*	LLC**WAFLQVW**SQS
P99		EED**FHHIREW**GNH

Heterogeneity of mCD1-autoreactive T cells

Numerous mCD1-reactive T cells and hybridomas have been described, and most of them apparently are autoreactive for mCD1 stimulators *in vitro* (48, 55, 64–66). The physiologic significance of these autoreactive T cells, some of which express relatively invariant TCRs, is a subject of intense study.

We have recently shown a striking degree of heterogeneity in the ability of mCD1-autoreactive T-cell hybridomas to respond to different mCD1$^+$ cells (55). We have proposed that this heterogeneity reflects the requirement for these cells to react with an autologous ligand presented by mCD1, and that there is a diverse set of such ligands, which could be either peptides or glycolipids. The mCD1-autoreactive T-cell hybridomas are derived from two sources. Some are from thymocytes selected for their expression of NK1.1 and other markers prior to cell fusion; as such, they are representative of NK T cells. NK T cells are a specialized subset of lymphocytes, representing 4% of splenocytes, 10 to 20% of mature thymocytes, and up to 30–50% of liver- and bone marrow-derived T cells (1). In addition to their intermediate level of TCR expression and their activated/memory-like phenotype, NK T cells are distinguished by their expression of receptors of the NK lineage, particularly NK1.1 (CD161) (1, 67). The majority of NK T cells use either the Vβ8, Vβ7 or Vβ2 chain paired with an invariant Vα14Jα281 rearrangement, thereby considerably restricting the diversity of their TCR repertoire (50). A homologous T-cell subset is found in humans. These T cells express a TCR containing Vα24JαQ and Vβ11 TCR , the homologs of mouse Vα14 and Vβ8, respectively (68, 69). Recently, it has been shown that some of these Vβ11/Vα24 T cells react with human CD1d, the closest human homolog of mCD1 (70). The second set of mCD1-autoreactive hybridomas that we have analyzed are derived from the residual CD4$^+$ population in class II-deficient mice (48). It is not certain if the cells that gave rise to these mCD1-autoreactive hybridomas were NK T cells (i.e. NK1$^+$), but, interestingly, none of them were Vα14$^+$.

By testing a panel of ten mCD1-autoreactive hybridomas, we found that each of the hybridomas has its own reactivity pattern against a series of mCD1-transfected thymocytes, fibroblasts, macrophages and B-cell lines, and suspensions of bone marrow-derived cells analyzed *ex vivo* (55) (L. Brossay, unpublished data). Some of the data from this analysis are summarized in *Table 2*. A few of the T-cell hybridomas react preferentially either to thymocytes or to splenocytes, despite a similar level of expression of mCD1 in these two cell types, whereas other hybridomas do not respond differentially to cells from these two organs (*Table 2*) (55). In addition, low numbers (3×10^4) of dendritic cells isolated from the spleen were able to induce a high level of IL-2 release from two hybridomas (2C12, 1A12), whereas high numbers of total splenocytes were relatively ineffective or weak stimulators (*Table 2*). Considering the number of antigen-presenting cells, dendritic cells were also better stimulators than splenocytes or thymocytes for hybridomas 24 and DN3A4-1-4. By contrast, hybridomas DN3A4-1-2 and 3C3 reacted poorly to dendritic cells. The response to transfected cells also does not correlate with the mCD1 expression level. For example, DN3A4-1-2 reacts much more strongly to untransfected BCL1 cells than to CD1.1-transfected A20 cells, while hybridoma 24 shows the opposite pattern, despite very high levels of mCD1 expression by both of these B cells.

To further analyze the requirements of these mCD1-autoreactive T cells, we asked whether the endosomal localization of mCD1 is required for their stimulation. We have recently demonstrated that the disruption of the mCD1 endosomal localization signal affected partially the reactivity of only one out of five mCD1-autoreactive T cells (55). Analysis of the reactivity of additional hybridomas shows that, out of ten hybridomas, only two are significantly affected by the disrup-

Table 2. Heterogenous response of different mCD1-autoreactive T-cell hybridomas to different mCD1-expressing cells

Hybridoma	TCR	A20 1×10^5	A20 mCD1 1×10	BCL1 1×10^5	Thymocytes 3×10^5	Splenocytes 3×10^5	Dendritic cells 3×10^5
DN3A4-1-2	α14/β8	0.25	6	49	4	0.6	1.6
2C12	α14/β8	0.7	102	124.7	4.8	13.9	55.4
1A12	nd	0.2	127	118	5.2	4	119
2-4	α3.2/β9	0.1	45	0.4	0.3	2	4.5
3C3	α14/β8	0.1	5.3	5.8	0.2	0.1	0.1
DN3A4-1-4	α14/β10	0.1	4	7	2.3	2.4	5.5

For the stimulation assays, 5×10^4 T-hybridoma cells per well were cultured in the presence of the number of antigen-presenting cells indicated in each column. After 20 h, IL-2 release was evaluated in a sandwich ELISA using rat anti-mouse IL-2 mAbs.

tion of the mCD1 endosomal localization signal (55) (L. Brossay, unpublished data). Interestingly, these two hybridomas were derived from NK T cells in the thymus, and they expressed a Vβ8/Vα14 TCR, although this requirement was not found for all of the Vα14$^+$ T cells. Based upon these data, we hypothesize that some of the autologous ligands bound to mCD1 molecules required for the stimulation of the autoreactive T cells are derived from endosomes, and that some are derived from other locations. Furthermore, we conclude that, even among the cells with an invariant α chain in their TCR, there is some degree of heterogeneity in terms of mCD1 autoreactivity which could be based upon the use of different Vβ gene segments or the diversity in the V-J junctional sequences of the β chain.

mCD1 can present glycolipids

Although CD1-restricted human T cells specific for glycolipid antigens have been described by a number of investigators (17, 19, 21), mouse T cells reactive with glycolipids have been only recently characterized by us and others (20, 71). Indeed, among several compounds tested, including lipoglycan antigens such as lipoarabinomannan and phosphatidylinositol mannoside, which have been shown to either bind to or be presented by hCD1b (72), only the synthetic glycolipid α-galactosylceramide (α-GalCer) was able to activate mouse splenic T cells *in vitro* (71) (N. Burdin, unpublished data). Furthermore, using Jα281-deficient mice that cannot make the invariant Vβ8/Vα14Jα281 TCR, or TCR-transgenic mice in which this is the only antigen receptor, Kawano et al. showed that it is the Vα14 T cells which respond to α-GalCer (20). Using both mCD1 transfectants and mCD1-deficient mice, we extended these findings by demonstrating that mCD1 is required for such α-GalCer-specific immune responses (71). We tested the panel of mCD1-autoreactive T-cell hybridomas described above for their ability to respond to α-GalCer. This was done using mCD1$^+$ APC, which did not give a high background of spontaneous autoreactivity in the absence of added ligand (see *Table* 2). The results of this analysis showed that both the T-cell-mediated immune response and the cytokine production induced by α-GalCer are associated specifically with the Vα14Jα281 rearrangement. The differential ability of mCD1-autoreactive hybridomas to respond to α-GalCer are further evidence for the heterogeneity of these T cells, and it is consistent with the autologous ligand requirement for these cells that we have hypothesized. The data suggest therefore that the diverse set of autologous ligands for the mCD1-autoreactive T cells could be lipids. Although the α-GalCer has only been found at low levels in mammalian cells, it is not certain if this compound is acting as a mimic for an autologous ligand that, when bound to mCD1, can stimulate NK T cells.

It has been proposed that the early expression of IL-4 by NK T cells could stimulate Th2-type immune responses (73–76), although this is not a requirement for Th2-mediated immune responses (77, 78). Furthermore, it is clear from our studies and from others that NK T cells can, under some circumstances, secrete large amounts of IFN-γ in addition to IL-4 (79–81). Functions for NK T cells in the regulation of autoimmune disease and the surveillance for tumors have also been proposed (82–88). With regard to autoimmune disease progression, the strongest evidence favors a connection between NK T cells and diabetes. The NOD mouse shows decreased numbers of NK T cells (84), and the ability of NK T cells in humans to secrete Th1 cytokines correlates with disease progression in humans (88). NK T cells were also found to be an essential target of IL-12, which induces their activation and enhanced cytotoxic activity (89–92). In addition, Vα14$^+$ NK T cells have been recently shown to be required in IL-12-mediated rejection of tumors (87), directly implicating the activation of these cells, and perhaps their cytotoxic activity, in the prevention of tumor metastases.

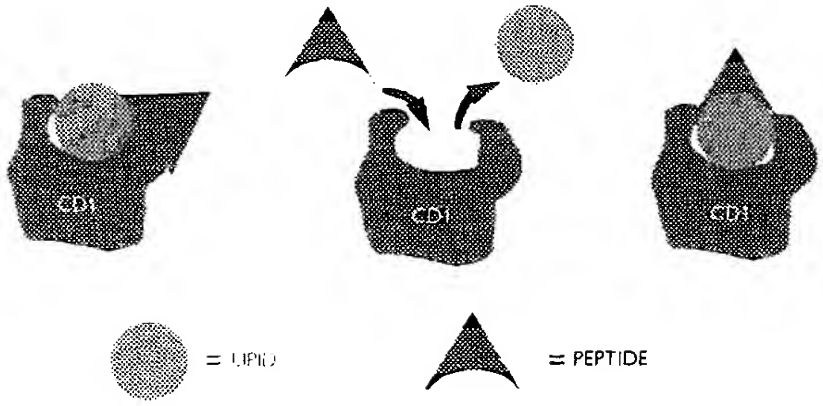

Fig. 4. Three models for peptide and lipid antigen binding to mCD1 molecules. Left: lipid containing antigens bind in the groove and peptides outside of the groove. Center: peptide and lipid antigens compete for binding in the mCD1 groove. Right: lipid antigen in the mCD1 groove forms part of the peptide antigen-binding site.

How does mCD1 bind to peptide and lipid antigens?

Several models may be considered to explain the interaction of CD1 with both glycolipids and peptides. Three models are outlined in Fig. 4, including one in which lipid antigen binds in the groove of CD1 and peptide outside of it, one in which both lipid and peptide antigens bind to the groove, and the possibility that a lipid forms part of the binding site for the peptides with a hydrophobic binding motif. We have not depicted a fourth logical possibility, namely that lipid binds outside of the groove and peptide inside in the groove. This goes against our expectation that the very hydrophobic acyl chains of lipid-containing antigens will be located in the two large pockets of the hydrophobic mCD1 groove, and the data suggesting that the electron dense material in the binding groove of soluble CD1 molecules obtained from insect cells appears to be lipid in nature (B. Segelke & I. Wilson, personal communication). Although the peptide has a hydrophobic binding motif, we also consider it unlikely that peptide and lipid compete for binding to the groove. The results from *in vitro* inhibition studies show that the peptide-specific T cells are poorly inhibited by anti-mCD1 mAbs (18, 22) (S. Tangri, unpublished data), whereas the stimulation of T cells by α-GalCer is completely inhibited by the same mAbs (20, 71). This suggests that the peptide ligand, but not the lipid-containing ligand, may be interfering with the binding of the mCD1 mAb to mCD1, although other interpretations of this data are possible. Second, preliminary data suggest that excess peptide does not compete for presentation of α-GalCer to Vα14$^+$ T-cell hybridomas, and, similarly, that excess α-GalCer does not compete for presentation of ovalbumin-derived peptides to conventional CD8$^+$ T cells (S. Tangri & N. Burdin, unpublished data). The most viable model is therefore the one depicted on the left in Fig. 4, in which the peptide binds to a site outside the groove. If this occurs, this site must be in part hydrophobic in nature in order to interact with bulky aromatic and aliphatic amino acids. Alternatively, the model depicted on the right in Fig. 4 could be correct, in which lipid deep in the mCD1 antigen-binding groove provides part of the peptide-binding site. If lipid were required for CD1 folding, transport and eventual peptide binding, this suggests that somewhat more of the bound antigenic peptide on CD1 could be exposed above the surface of the antigen-presenting molecule, when compared to peptides bound to class I or to class II molecules.

Evolution of CD1 molecules

The presence of CD1 molecules throughout the mammalian class, and the lack of polymorphism of these antigen-presenting molecules, suggests that CD1 may carry out an important function and that this is distinct from the functions of MHC class I and class II molecules. If presentation of lipoglycan antigens were a unique attribute of CD1 molecules, this could explain the maintenance of CD1 during mammalian evolution. In earlier reviews (7, 10), we also proposed that the presentation of peptides would more likely lead to polymorphism than the presentation of lipoglycans. This would be so because short peptides could readily accommodate mutations that would inhibit presentation, without necessarily inhibiting the function of the intact protein. The diversity and potential for rapid change in dominant peptide determinants could therefore drive the generation of MHC class I and class II polymorphism. By contrast, glycolipids are the end result of a complex biosynthetic process, and therefore they are not as capable of changing rapidly during evolution. Furthermore, because CD1 molecules have a broad specificity for the presentation and binding of different kinds of lipids with two acyl chains, or a single branched acyl chain, including mycolic acids, phosphatidylinositols and

ceramides (17, 19, 21, 72), it would be difficult for microorganisms to create mutations that would completely abolish these types of structures.

If CD1 molecules were selected primarily for their ability to present non-peptide antigens, what is the importance of the presentation of peptides? Do the peptides with a hydrophobic binding motif only represent a mimic of lipids? The recent data from the ovalbumin system clearly indicate that CD1 can present peptides from processed proteins that are acquired in an endosomal compartment, arguing that this peptide presentation process must have some physiologic relevance, even if CD1 molecules are selected in evolution primarily for their binding to lipid containing antigens. Interestingly, the CD1 peptide-binding motif is most frequent in viruses, particularly those with RNA containing genomes. Among pathogenic microorganisms, viruses are unique in that they lack glycolipids, or their envelope contains only host-derived lipids and carbohydrates. Because CD1-mediated immune responses may in general be highly protective, it is possible that CD1 molecules are selected dually during evolution both for their ability to present lipoglycans from bacteria and perhaps also from protozoan parasites, and also for their ability to present peptides, particularly from viruses.

What is the selective advantage of CD1-mediated T-cell responses?

The main driving force behind the evolution of the vertebrate immune system is host defense against pathogenic microorganisms. The strongest data indicating a possible role for CD1-mediated responses in host defense come from studies of human CD1b- and CD1c-restricted T cells (17, 19, 21, 93, 94). CD1b- and CD1c-restricted T cells can recognize lipoglycan antigens from pathogenic microorganisms, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, and, in at least one case, such T cells have been retrieved from the lesions of a leprosy patient (19). Once activated, lipoglycan antigen plus CD1b-reactive T cells can secrete Th1-type cytokines (19). Furthermore, the cytolytic activity of CD1b-restricted T cells has been recently shown to be lethal for the bacteria as well as the target cell (95). Although correlative in nature, taken together these data strongly suggest a role for CD1-reactive T cells in the protective cell-mediated immunity against bacteria in humans.

There is a wealth of data, however, suggesting that many CD1-reactive T cells do not recognize microbial antigens, implying that these cells may not participate directly in the recognition and elimination of microbes. A significant fraction of the CD1-reactive T cells isolated from mice and humans are in fact CD1-autoreactive. Most notably, this CD1 autoreactivity holds true for the mouse NK T-cell population. Although it is not known if NK T cells are truly mCD1-autoreactive *in vivo*, they do have a cell-surface phenotype characteristic of memory or activated T cells, including high levels of CD44 and CD69 and low levels of CD62L (1). The results from several studies suggest that the interaction of NK T cells with CD1 may be the most important function for these molecules. First, as noted above, NK T cells are abundant in mice, particularly in liver, bone marrow and spleen. Second, this system seems to be highly conserved. Mice do not have homologs of human CD1a, CD1b or CD1c, and the comparative studies carried out so far, albeit in relatively few species, suggest that molecules related to mCD1 and its human CD1d homolog are the most widely distributed in mammals. In addition, the system of invariant TCR recognition of CD1 molecules is highly conserved between mice and humans (68–70, 96).

While the significance of this widespread autorecognition of CD1 molecules is not certain, the results from a number of studies suggest that NK T cells play an important immunoregulatory role. As postulated originally by others, through the secretion of large amounts of cytokines, NK T cells may help to direct polarization of immune responses in either the Th1 or the Th2 direction (73–76, 79–81). Therefore, NK T cells could play an important role in the clearance of pathogens, as well as in the regulation of antiself and antitumor responses. Because mCD1 molecules are constitutively present, autorecognition of mCD1 by NK T cells is likely to require an mCD1-bound ligand that is induced only under some circumstances. We further speculate that this autologous ligand is likely to be a lipoglycan, perhaps with a good degree of similarity to α-GalCer. The conditions governing the presentation of this autologous ligand by CD1 molecules remain to be determined, but we speculate that stress, apoptosis or necrosis are candidate conditions for the induction of ligand/CD1 complexes capable of activating NK T cells. Of course, there are no data which rule out the alternative hypothesis, namely that the NK T cells are reactive to some common microbial products, and that the autoreactivity observed is only of lesser significance. Characterization of the natural ligand(s) recognized by NK T cells is likely to shed light on this question, and it could have significant impacts on our understanding of the evolution of antigen-presenting function and the regulation of immune responses.

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